

# ***U.S. PATENT APPLICATION***

***Inventor(s):*** Peter Lewis Keeling  
Frances Katz  
Ming-Tang Chang  
Richard Hauber  
Robert Friedman

***Invention:*** NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM

***NIXON & VANDERHYE P.C.  
ATTORNEYS AT LAW  
1100 NORTH GLEBE ROAD  
8<sup>TH</sup> FLOOR  
ARLINGTON, VIRGINIA 22201-4714  
(703) 816-4000  
Facsimile (703) 816-4100***

## ***SPECIFICATION***

## NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM

### FIELD OF THE INVENTION

5 The invention relates to a transgenic or mutated plant having genomic material which alters the normal starch synthesis pathway within the plant. More specifically, the present invention relates to a plant having a genotype which creates new forms of starch in significant quantity. Particularly, the invention relates to grain having an embryo with a genotype heterozygous for two or more wild type genes (for example, 10 Aa/Bb) and an endosperm having a genotype heterozygous for such genes (for example, AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB) and the starch produced therefrom.

Such grain are produced by pollinating a plant having a genotype homozygous 15 recessive for at least one gene and wild type for another gene (for example, aa/BB) with pollen from another plant having a genotype homozygous recessive for at least one other gene and wild type for the other gene (for example, AA/bb).

### BACKGROUND OF THE INVENTION

20

Most plants produce and store starch. These plants have a starch synthesis pathway for starch production. The amount of starch produced varies with the type of plant. The most commonly known starch producing plants are the cereal grains. These cereals include rice, maize, sorghum, barley, wheat, rye, and oats. Additionally, the 25 potato family, including the sweet potatoes and certain fruits, like the banana, are known as starch producing.

Starch is an important end-product of carbon fixation during photosynthesis in leaves and is an important storage product in seeds and fruits. In economic terms, the 30 starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories.

Starch from plants is used in various ways. For example, it can be extracted and used for cooking and food processing. Starch can be left in the grain or plant and  
35 used for animal and human consumption. Starch can also be used in the distillation process for processing alcohols, for example, starch can be converted into ethanol. Additional starch can convert to high-fructose syrup and other industrial components.

Starch is defined in the dictionary as a granular solid which is chemically a  
40 complex carbohydrate which is used in adhesives, sizes, foods, cosmetics, medicine, etc. More generally, starch is comprised of amylose and amylopectin. Amylose and amylopectin is synthesized in the plastid compartment (the chloroplast in photosynthetic cells or the amyloplast in non-photosynthetic cells). Different plants generate differing proportions of amylopectin and amylose. Furthermore, the different  
45 branching patterns of amylopectin and different chain lengths of amylose and amylopectin chains gives rise to different starch properties. Thus, the fine structure of amylose and amylopectin is different in different plants so that the branching patterns and chain-lengths vary considerably resulting in new and novel properties which are useful in different applications. Until now there have been four ways of making  
50 starches with special properties: (i) using starches extracted from different plant species, (ii) using starches extracted from mutant lines of particular plants, (iii) using natural and mutant starches which had been chemically modified, and (iv) using natural and mutant starches which had been physically modified. In all cases the new starches were valuable because of the special properties provided for by the new starch type.

55

It is known that mutant genes in plants affect the properties of the starch. A variety of starch related mutant genes in maize have been identified and some have been cloned. These mutant genes were named according to the physical appearance (phenotype) of the maize kernel or the properties of the starch. These recessive mutant  
60 genes include waxy (wx), sugary (su) [which includes but is not limited to sugary-1 (su1), sugary-2 (su2), sugary-3 (su3), sugary-4 (su4)] dull (du), amylose extender (ae),

horny (h), shrunken (sh) which includes, but is not limited to, shrunken-1 (sh-1), shrunken-2 (sh-2). Some of these recessive gene mutants produce an isoform of a known enzyme in the starch synthesis pathway. The recessive mutant alleles of these  
65 genes result in a complete or nearly complete reduction in the activity of a specific isoform of one enzyme (hereinafter defined as complete reduction of enzyme isoform activity) in the pathway when homozygous in a plant or when expressed in sufficient levels in a transgenic plant. This change in the starch synthesis pathway causes the formation of starches with different properties.

70

Several crop varieties are known which produce different types of starch. The type of quality of starch makes it suitable for certain purposes, including particular methods of processing or particular end-uses. Naturally-occurring maize mutants produce starches of differing fine structure suitable for use in various food products and  
75 other applications. Although known mutants produce altered starch, some of these lines are not suitable for crop breeding and/or for the farmers' purposes. For example, they can give relatively poor yields, and/or are difficult to process and/or can have poor germination.

80 In order to generate different starches, single and double mutant plants have been bred. A single mutant is a plant that is homozygous for one recessive mutant gene. For example, waxy maize, waxy rice, waxy barley, and waxy sorghum have the homozygous mutant waxy (wx) gene. Whilst starches from waxy genotypes have very little or no amylose, another mutation known as amylose extender (ae) results in starch  
85 with high amylose. A double mutant is a single plant that has homozygous (or full expression) of two recessive mutant genes. For example, the wxfl1 double mutant is taught in U.S. patent 4,789,738. Many other novel starches have been provided in other starch patents in which double or triple mutants are generated (for example, US Patent Serial Numbers: 4789557, 4790997, 4774328, 4770710, 4798735, 4767849, 4801470,  
90 4789738, 4792458 and 5009911 which describe naturally-occurring maize mutants producing starches of differing fine structure suitable for use in various food products).

The present invention is highly surprising in light of these applications because it produces altered starch and does not require double or triple mutants.

95 Normal starch is defined as starch which is not chemically modified (by people) or which is produced from a plant that has the expected genes (wild type) regulating the starch synthesis pathway. For ease of reading, double lower-case letters, for example aa, shall refer to a homozygous recessive mutant gene, double upper-case letters, for example AA, shall refer to a homozygous non-mutant gene (wild type), and  
100 one upper-case and one lower-case letter, for example Aa, shall refer to a non-homozygous set of genes, one mutant, one non-mutant. Different letters in the same size shall mean different genes; "aa/bb" would be a double mutant; "aa/bB" would be a single homozygous mutant gene and a heterozygous mutant gene in the genome of the plant. For purposes of this application, the order of any three letters on one side of the  
105 slash can be interchanged and will not define the parent that donated the gene. For example, AAa/bbB is defined to be equivalent to aAA/bBb, AaA/Bbb, AaA/bBb, aAA/Bbb, and the like.

Although maize plants and the embryo are diploid, maize endosperm is triploid.

110 The endosperm genotype has two gene doses which are inherited from the female plant portion and one gene dose which is inherited from the pollen or male plant portion. Thus, if a single mutant plant "aa" is used as the female and crossed to a non-mutant plant "AA" male, then the endosperm in the kernel of this female plant would be "aaA". If a non-mutant plant "AA" is crossed to a mutant plant "aa" with the non-  
115 mutant as the female, the endosperm on the kernel of the female plant will be "Aaa", because two gene doses come from the female and one from the male plant. Classic teaching is that the mutant gene is recessive and the non-mutant is dominant; therefore, the starch produced by a plant having the following gene doses in the endosperm "aaA" or "AAA" or "AAa" results in the normal starch in the expected amounts. However,  
120 the endosperm of a homozygous mutant plant "aa" acting as the female crossed to a homozygous mutant plant "aa" acting as the male plant results in the endosperm having

the gene dosage "aaa". This endosperm gives starch with different properties from normal starch. Likewise, the starch from a double mutant having of an endosperm which is "aaa/bbb" shows differences in starch properties from normal starch. These  
125 starch differences are useful in that they can replace chemically modified starches or be used with or in foodstuffs or as grain in alcohol production or in general starch industrial applications.

Clearly, it appears that production of grain having starch with different physical  
130 properties of starch requires the crossing of two mutated plants to generate grain which is homozygous recessive for both genes. Mutant plants are less predictable than standard plants.

There is a recurrent problem with the production of grain and extraction of  
135 starch from double mutant hybrids and/or inbreds and some single mutants. The amount of starch produced is usually less than the amount of starch produced by the non-mutant plant, there is also a loss in starch granule size and/or starch granule integrity. This problem with known double mutant lines which produce structurally-altered starch in which the quantity of starch produced in the crop is relatively low can  
140 result in poor germinability of the seed. Furthermore, the reduced starch yield of the seed appears to be unavoidable since the mutations cause the normal starch synthesis functioning of the cells to be disrupted. There remains a need for a way to produce grain having structurally altered starch structurally altered starch or altered properties without a significant loss of yield or reduced starch granule size or integrity.

145

#### SUMMARY OF THE INVENTION

A first object of the present invention is to provide a method of developing hybrid plants having altered complex carbohydrate content of the grain which does not  
150 require the crossing of double mutant inbreds.

An object of the present invention is to provide plants that produce grain having altered starch properties.

155 Another object of the present invention is to provide transgenic plants that produce grain having altered starch properties.

Yet another object of the present invention is to provide a maize plant that produces both altered starch and larger quantities of starch than the associated mutant  
160 plants produce.

Still another object of the present invention is to provide new plants which contain genes which produce incomplete reduction of the activity of at least two isoforms of the specific enzymes in the starch synthesis pathway of the plant.

165

A further object of the present invention is to provide maize plants which have endosperms with the genotype "AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB".

Yet another object of the present invention is a plant producing the following  
170 endosperm genotype "wxwxWX/AeAeae".

Still an additional object of the present invention is to provide the altered starch which can be produced by plants having the genotype "AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB".

175

Yet another object of the present invention is to provide novel uses of the starch obtained from the Maize plants of the present invention.

The present invention broadly covers a method of producing intermutants  
180 generally which have the endosperm genotype of AAa/bbB and certain intermutants including endosperm which is waxy, waxy, amylose extender (wxwxWx/AEAEae).

The method of producing grain with altered starch qualities includes the steps of planting the female acting parent which is capable of flowering. The female parent having substantially complete reduction of at least one specific isoform enzyme in the starch synthesis pathway. This can be by a homozygous recessive mutant or by the partial down regulation of the wild type gene through the use of a cloned gene using techniques generally known as antisense or co-suppression or sense-down regulation. Additionally the female has incomplete reduction of at least one specific isoform enzyme in starch synthesis pathway. This can be by a heterozygous recessive mutant gene or partial down regulation. Regardless of how the female is produced it should only act as the female portion. To assure this a step includes eliminating the first parent's capability to produce pollen. The method includes the step of pollinating the female acting parent with the pollen of the male acting parent which is a non-mutant parent. Harvesting the grain produced by said first parent. Additionally, the method can include the extraction of starch from the grain.

This invention also encompasses a plant having genomic material which includes genes which give incomplete reduction of the activity of at least two specific isoforms of the enzymes in the starch synthesis pathway of said plant. And the starch it produces which has altered structure when compared with the starch formed by a similar plant as described but which comprises genomic material which does not form isoforms of the enzymes in the starch synthesis pathway of the plant.

A plant which forms said starch in grain such as cereal grains. Grain produced by a female plant having for example a waxy genotype (wxwx) crossed with a male plant having for example an amylose extender genotype (aeae) in which the genotype of the endosperm of the grain is wxwxWx/AeAeae.

In other words, the present invention is a starch producing plant comprising genomic material which includes genes which give incomplete reduction of the activity of at least two specific isoforms enzymes in the starch synthesis pathway of said plant



whereby said plant produces substantially more starch than said plant would produce if said genes gave complete reduction of the activity of the same two specific isoforms of the enzymes within the starch synthesis pathway.

215

Grain having an endosperm that has two genes which contain one gene dose of recessive mutant gene and two doses of wild type; and, having two gene doses of recessive mutant and one dose of wild type. Within this description the invention encompasses grain having endosperm genotypes of wxwxWx/AeAeae, or  
220 aeaeAe/WxWxwx, or wxwxWx/DuDudu, or duduDudu/WxWxwx, or aeaeAe/DuDudu, or duduDudu/AeAeae, or wxwxWx/SuSusu, or susuSu/WxWxwx, or aeaeAe/SuSusu, or susuSu/AeAeae, or duduDudu/SuSusu, or susuSu/DuDudu and the like.

The starch from a grain having a genotype of wxwxWx/AeAeae. The starch  
225 from a grain having a genotype of Aeaeae/WxWxwx.

A female plant having a diploid genotype of aa/BB and having a triploid genotype of aaA/BBb where a is a recessive mutant gene and A is the wild type gene, and b is a recessive mutant gene and B is the wild type gene such that the starch is  
230 altered from the normal starch where a and b can be selected from ae, wx, sh, bt, h, su, fl, op and B and A can be selected from Ae, Wx, Sh, Bt, H, Su, Fl, Op.

The starch obtained in accordance with the present invention produces a strong resilient gel which clears from the mouth uniquely fast. The starch of the present  
235 invention produces a gel with a unique and distinctive texture compared to conventional starches. The unique and distinctive texture makes the starch of the present invention suitable as a replacement for conventional gelling gums such as natural gums and gelatin, in whole or in part in food formulations. The starch of the present invention has also been found to produce a more resilient gel than common  
240 starch. Furthermore, it has been found that cornstarch produced from maize produces a gel which has improved clarity compared to a gel made from a common starch. Such

improved clarity is visible to the human eye and lends itself to a more appetizing foodstuff.

245

**BRIEF DESCRIPTION OF THE DRAWINGS**

The present invention will now be described, by way of illustration, by the following description and examples with reference to the accompanying drawings of which:

250

FIG 1 is a graph of enzyme activity for different gene dosages of a single mutant;

255

FIG 2a is a graph of the DSC scan of waxy, amylose extender and common maize;

FIG 2b is a graph of the DSC scan of a double mutant (aeae/wxwxwx);

260

FIG 2c is a graph of the DSC scan of starch from an intermutant (aeae/wxwxwx).

FIG 2d is a graph of the DSC scan of starch from another intermutant (wxwxWx/AeAeae).

265

FIG 3a is a graph of Brabender data of common starch in various pH.

FIG 3b is a graph of Brabender data of waxy starch in various pH.

FIG 3c is a graph of Brabender data of 70% amylose starch in various pH.

270

FIG 3d is a graph of Brabender data of double mutant starch in various pH.

- FIG 3e is a graph of Brabender data of a first intermutant starch in various pH.
- 275 FIG 3f is a graph of Brabender data of a second intermutant starch in various pH.
- FIG 4a is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of branching enzyme I.
- 280
- FIG 4b is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of branching enzyme II.
- 285
- FIG 4c is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of bound starch synthase (waxy).
- 290 FIG 4d is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels soluble starch synthase.
- FIG 5 is a plot of the elastic modulus ( $G'$ ) over time comparing a gel made from the starch of the present invention to a gel made from an aewx starch and a gel made from a waxy (wx) starch; and
- 295
- FIG 6 is a plot of the elastic modulus ( $G'$ ) plotted against strain for both the present invention and an aewx starch.
- 300

## DETAILED DESCRIPTION OF THE INVENTION

Broadly then the present invention is an improved crop line which has manipulated expression of at least two starch-synthesizing enzymes which alter the  
305 amount and type of starch, and, consequently, alters the grain produced by the plant.

It has been discovered that plants which contain at least two genes which partially down regulated or reduce the activity of specific isoforms of enzymes in the starch synthesis pathway will surprisingly produce significant amounts of starch in the  
310 grain and will produce altered starch types.

Specialty maize or mutant plants differ from "normal" maize because of its altered endosperm. The changed endosperm gives rise to a high degree of starch branching, or changed sugar content, or different kernel structure. The endosperm of  
315 course is formed by the sperm and ovule, and the selection of both parents effects the endosperm's makeup.

The present invention can be formed by two principle methods. The invention can be formed within a selected crop species by the use of mutant breeding. And the  
320 invention can be formed in various plants by the use of transformation of the plants with genes which partially down regulate two or more enzymes in the starch synthesis pathway. More particularly, down regulation of one of the isoforms of the enzyme in the starch synthesis pathway to approximately 1/3 of the normal activity and 2/3 of the normal activity in the other isoform enzyme or down regulation of both isoform  
325 enzymes in the starch synthesis pathway approximately 2/3 of the normal activity. Each of these methods has its own advantages.

First, the use of mutants to develop unique grain and starches in cereal crops is widely known. However, the present invention is highly unique and surprising because

330 it was expected to produce grain having normal starch characteristics. The following table explains how unexpected the present invention is.

**TABLE 1**

335	Genotype of Parents		Genotype of endosperm	Type of Starch	
	(female 1st)			Starch	Yield
	Wild-Type	AA*AA	AAA	Normal	Normal
340	Gene dosage	AA*aa	AAa	Normal	Normal
		aa*AA	aaA	Normal	Normal
	Single mutant	aa*aa	aaa	Altered	Lowered
345	Double mutant	aa/bb*aa/bb	aaa/bbb	Altered	Lowered

**PRESENT INVENTION - EXPECTED RESULTS**

350	Intermutant	aa/BB*AA/bb	aaA/BBb	Normal	Normal
		AA/BB*aa/bb	AAa/BBb	Normal	Normal
		aa/bb*AA/BB	aaA/bbB	Normal	Normal
		aa/bb*aa/BB	aaa/bbB	Normal	Normal
		aa/BB*aa/bb	aaa/BBb	Normal	Normal

**PRESENT INVENTION - ACTUAL RESULTS**

355	<i>Intermutant</i>	<i>aa/BB*AA/bb</i>	<i>aaA/BBb</i>	<i>Altered</i>	<i>Medium to</i>
		<i>AA/BB*aa/bb</i>	<i>AAa/BBb</i>	<i>Altered</i>	<i>high ~ 70%</i>
		<i>aa/bb*AA/BB</i>	<i>aaA/bbB</i>	<i>Altered</i>	<i>of normal</i>
		<i>aa/bb*aa/BB</i>	<i>aaa/bbB</i>	<i>Altered</i>	
		<i>aa/BB*aa/bb</i>	<i>aaa/BBb</i>	<i>Altered</i>	
360					

aa = mutant gene (homozygous)

AA = wild type (or non-mutant homozygous gene)

\* = signifies a cross-pollination between two varieties

365 aa/bb = two mutant genes (both homozygous)

Clearly, since the genotype of the endosperm of the present invention shown in the table did not have completely recessive genes, the starch yield and structure was expected to be normal. In fact, according to the present invention, the grain does not  
370 evidence normal starch structure altered starch. Historically, when altered starch is produced only small quantities are usually produced. The altered starch of the present invention was surprisingly produced in larger than expected quantities. Additionally, production of this starch is much simpler than the production of double mutant crops. Previously only single mutant hybrids have been used extensively for large-scale starch  
375 production. Previously, to develop the double mutants, both parents had to carry both mutations which requires significant research and development efforts and results in poor starch yield and poor seed germinability. Heretofore, only small-scale production of the double mutants has been possible.

380 The present invention encompasses a method of producing grain with altered starch qualities which includes the steps of planting a parent which is capable of flowering, this parent having substantially complete reduction of at least one specific isoform of an enzyme (A) in the starch synthesis pathway and having no reduction of at least one other specific isoform of an enzyme (B) in the starch synthesis pathway. The  
385 other parent has no reduction of one isoform of an enzyme (A) and substantially complete reduction of at least one other specific isoform of an enzyme (B) in the starch synthesis pathway. It is then necessary to eliminate said first parents capability to produce pollen and allow pollination to proceed from said second mutant parent, and finally harvesting the grain produced by said first parent. Additionally, the method can include the  
390 extraction of starch from the grain and using said starch as a specialty starch for a variety of uses for which it is shown to be valuable.

In order to prepare a sol in accordance with the present invention, a slurry is prepared which comprises water and an effective amount of starch of the present invention  
395 and the sol subject to a cooking step to form a paste. Generally, cooking entails raising

the temperature of the slurry to above about the gelatinization temperature of the starch and subjecting the starch to enough shear such that the granules rupture and a paste is formed. It is not necessary that all the granules rupture. Preferably, the sol contains the starch of the present invention in the amount of about 1 to about 20% by weight total sol.

400 The slurry is cooked at a temperature of about 90° C and above to provide thickening characteristics prior to adding to the foodstuff. Cooking time is about 10 minutes. The sol in accordance with the present invention need not be cooked if the starch has already been subjected to a process which makes it cold water swellable. Cooking generally comprises raising the temperature of an aqueous slurry of the starch of the present

405 invention to the gelatinization temperature of the starch and subjecting the starch to shear such that the starch granules rupture and form a paste.

A sol or a thickener composition of the starch of the present invention is added to a foodstuff in a conventional manner in order to provide the benefits of the starch of the

410 present invention to the foodstuff.

In order to prepare the thickened foodstuff, a sol made in accordance with the present invention is combined with a foodstuff and the composition is cooked to the necessary degree to provide a thickened foodstuff. Conventional mixing is employed to

415 combine the sol with the foodstuff. Cooking of the sol and foodstuff composition is also carried out in a conventional manner.

Alternatively, starch of the present invention is mixed with the foodstuff or a slurry comprising the starch of the present invention and water is mixed with a foodstuff

420 and the resulting mixture is cooked to the desired degree to obtain a thickened foodstuff. When the starch itself or a slurry containing the starch itself is mixed with a foodstuff, the resulting mixture must be cooked in order to provide a thickened foodstuff. The mixing as well as the cooking is accomplished in a conventional manner. Cooking is carried out at a temperature of about 90° C and above. Cooking time is about 10 minutes but may

425 vary depending on the amount of foodstuff present and the amount of shear that the mix is subject to during cooking.

Such a thickener composition can provide considerable economic advantage to the user. Those familiar with the art have long used a variety of gelling gums for their clean  
430 breaking texture. Application of the present invention have included but are not limited to gum candies, gelled desserts, glazes and spreads and can be used to replace conventional gelling gums such as kappa carrageenin, agar, pectin, or gelatin. These conventional gelling gums can be quite expensive however, and have other disadvantages including the presence of off-flavors, lack of heat or acid stability, limited availability, or lack of Kosher  
435 approval. It has been found that the starch of the present invention can replace all or a portion of these conventional gelling gums.

In order to replace a gelling gum in food formulations, a weight ratio of about 1:1, starch of the present invention:gelling gum, can be employed. Larger or smaller amounts  
440 of the starch of the present invention may be used to replace a gelling gum. Such gelling gums include gelatin, pectin, carrageenin, gum arabic, tragacanth, guar, locust bean, zanthan, agar, algin and carboxymethyl cellulose.

Naturally, the starch of the present invention can be used in any food formulation,  
445 where there is a need to provide gel characteristics and a clean break from the mouth. For example, the starch of the present invention can be used in a food formulation which had heretofore employed a common starch, thereby providing the food with improved properties, i.e. clean break when compared to the same food formulation using a common starch.

450

The clean break of a gel made with the starch of the present invention is useful in a variety of food applications. The clean break of the starch gel has value in a variety of bakery applications, for example cream or fruit fillings for pies such as lemon, banana cream or Bavarian cream; and in low or reduced fat high solids fruit centers for cookies,



455 for example, in fig bars. The starch of the present invention also creates an improved texture in mousses, egg custards, flans and aspics.

The term starch as used in the specification and claims means not only the substantially pure starch granules as extracted from a starch bearing plant but also grain  
460 products of the starch granule such as flour, grit, hominy and meal.

The following examples of the present invention are given for illustrative purposes only. These examples are not intended to limit the type or the uses of the present invention. The present invention or the grain or starch or sugar thereof can be useful in,  
465 but may not be limited to, the preparation of foodstuff, paper, plastics, adhesives, paints, production of ethanol and corn syrup products.

#### Example 1

470 The physical properties of various embodiments of the present invention (similar genotypes are from different maize crosses). These tables display data well known to people skilled in the arts of evaluating new and novel starches. Data on moistures, and percent oil, protein, solubles and starch are  
475 useful in evaluating yield and milling potential. Starch DSC (Differential Scanning Calorimetry) data are valuable for evaluating starch cooking and gelatinization properties. Starch particle size data are valuable for making decisions on starch milling and separation properties. Brabender and starch paste  
480 data are most important for evaluating a new starch's potential for improved food applications where particular starch thickening and pasting and gelatinization properties are mostly desired. Such data when interpreted as a whole collection of information enable one skilled in the art to

485

decide whether to conduct further more detailed tests of the  
starches properties and potential.

Genotype	%Moisture	%Starch	%Protein	%Oil	%Solubles
aeae/wx	8.01	72.12	11.30	3.63	6.51
aeae/wx	8.00	71.34	11.74	3.79	6.41
aeae/wx	8.03	67.52	12.61	3.67	7.01
aeae/ae	8.13	60.11	14.59	6.60	10.14
su1su1/wx	8.18	69.66	12.09	4.03	6.91
dudu/wx	7.98	71.10	11.57	3.92	6.61
dudu/su1	7.90	70.53	11.67	3.93	6.81
flfl/o	7.78	70.23	11.60	4.23	6.59
su1su1/du	7.81	69.00	12.20	4.77	7.98
su1su1/ae	7.88	69.03	12.27	4.88	8.02
su1su1/su2	7.95	69.67	12.28	4.55	7.49

490

Table 2

## - Corn Data

<u>Entry</u>	<u>Background</u>	<u>S93</u> <u>Row</u>	<u>%</u> <u>Moisture</u>	<u>%</u> <u>Starch</u>	<u>%</u> <u>Protein</u>	<u>%</u> <u>Oil</u>	<u>%</u> <u>Solubles</u>
1	acaewx	4660	9.15	64.80	14.58	4.47	7.64
2	acaesu	4636	9.27	66.25	14.77	4.54	7.40
3	acaedu	4612	9.24	66.01	14.65	4.73	7.80
4	wxwxae	4600	9.07	65.72	13.91	4.38	8.14
5	wxwxsu	4648	9.06	66.03	14.79	4.08	7.83
6	wxwxdu	4624	8.77	65.32	15.62	4.27	8.23
7	susuae	4594	8.85	64.83	13.11	4.71	8.49
8	susudu	4672	8.73	65.39	13.20	5.01	8.35
9	susudu	4618	8.77	65.15	14.52	4.35	8.26
10	single ae	4582	9.14	60.52	15.19	5.95	9.29
11	single wx	4654	8.83	65.44	14.04	4.77	9.43
12	single su	4630	7.75	54.98	14.25	6.50	9.78
13	duduae	4588	8.97	69.26	12.30	4.89	6.85
14	duduwx	4666	8.88	70.43	11.25	4.40	6.96
15	dudusu	4642	9.48	70.43	13.48	4.67	6.92
16	single du	4606	8.50	65.54	12.02	6.03	9.33

## - Starch Data

<u>Entry</u>	<u>Background</u>	<u>S93</u> <u>Row</u>	<u>%</u> <u>Moisture</u>	<u>%</u> <u>Starch</u>	<u>%</u> <u>Protein</u>	<u>%</u> <u>Oil</u>	<u>%</u> <u>Amylose</u>	<u>L-max</u>
1	acaewx	4660	3.82	86.44	1.68	0.18	31.60	597.1
2	acaesu	4636	6.51	86.95	1.56	0.20	31.74	603.9
3	acaedu	4612	5.22	86.44	1.70	0.11	33.38	598.8
4	wxwxae	4600	7.33	87.89	0.39	0.09	21.10	601.1
5	wxwxsu	4648	7.27	85.87	1.32	0.09	24.68	591.7
6	wxwxdu	4624	7.31	86.07	1.12	0.10	24.57	595.2
7	susuae	4594	8.19	86.32	0.93	0.07	29.00	604.5
8	susudu	4672	8.09	87.16	0.52	0.08	27.86	599.6
9	susudu	4618	7.67	84.20	0.60	0.12	30.82	601.1
10	single ae	4582	7.16	83.31	1.37	0.15	65.55	602.1
11	single wx	4654	8.30	85.16	0.51	0.14	0.43	xx
12	single su	4630	6.82	80.92	5.08	xx	29.32	598.4
13	duduae	4588	6.58	84.31	0.62	0.17	29.86	xx
14	duduwx	4666	11.08	84.77	0.48	0.16	28.74	xx
15	dudusu	4642	8.48	87.34	0.62	0.16	26.07	xx
16	single du	4606	8.36	80.70	0.46	0.08	37.88	608.9

495

Table 2

## - (Starch Data)

<u>Entry</u>	<u>Genotype</u>	<u>% Moisture</u>	<u>% Starch</u>	<u>% Protein</u>	<u>% Oil</u>	<u>% Amylose</u>	<u>L-max</u>
1	ae ae / wx	9.56	83.84	0.63	0.05	29.44	593.1
2	ae ae / wx	7.96	85.94	0.70	0.05	27.89	596.6
3	ae ae / wx	11.71	82.54	0.82	0.05	42.54	595.1
4	ae ae / ae	6.09	84.17	1.24	0.14	63.60	602.4
5	su1 su1 / wx	4.37	90.00	0.56	0.09	28.36	600.0
6	du du / wx	5.95	88.50	0.61	0.09	27.98	596.9
7	du du / su1	6.93	82.66	0.69	0.10	29.98	600.8
8	fl fl / 0	9.24	82.72	0.93	0.14	29.29	596.8
9	su1 su1 / du	9.01	84.82	0.71	0.02	29.57	600.8
10	su1 su1 / ae	7.78	84.81	0.63	0.03	29.90	600.8
11	su1 su1 / su2	7.61	85.02	0.49	0.02	29.68	602.3
12	White waxy	18.34	94.94db	0.77	0.09	2.74	526.9

## - Starch DSC Data

<u>Entry</u>	<u>Genotype</u>	<u>Peak °C</u>	<u>Delta H J/g</u>	<u>Peak II °C</u>	<u>Onset °C</u>	<u>Endset °C</u>
1	ae ae / wx	67.7	11.67	100.0	62.6	76.6
2	ae ae / wx	66.5	10.33	96.8	61.9	74.8
3	ae ae / wx	74.6	10.00	100.8	68.8	83.4
4	ae ae / ae	81.0	11.83	xx	66.0	107.4
5	su1 su1 / wx	69.7	12.00	98.3	64.2	77.7
6	du du / wx	72.2	11.50	99.5	66.6	82.3
7	du du / su1	70.2	9.67	98.4	65.7	78.3
8	fl fl / 0	71.4	12.33	100.9	67.1	80.7
9	su1 su1 / du	68.8	7.83	99.5	63.2	79.1
10	su1 su1 / ae	68.1	10.33	99.8	61.9	78.3
11	su1 su1 / su2	67.4	12.00	96.1	61.3	79.5
12	White waxy	72.7	15.33	xx	66.1	82.6

## - Starch DSC Data

<u>Entry</u>	<u>Background</u>	<u>S93 Row</u>	<u>Peak °C</u>	<u>Delta H J/g</u>	<u>Peak II °C</u>	<u>Onset °C</u>	<u>Endset °C</u>
1	acaewx	4660	73.8	11.17	99.4	67.7	82.8
2	acaewx	4636	73.0	11.50	101.0	66.6	83.4
3	acaewx	4612	73.3	10.83	98.6	66.7	83.0
4	wxwsae	4600	72.9	12.83	99.9	67.4	81.7
5	wxwssu	4648	73.7	12.17	98.2	68.2	81.6
6	wxwsdu	4624	73.6	11.33	99.6	69.3	80.5
7	susuae	4594	72.0	10.33	97.7	67.2	79.7
8	susudu	4672	71.9	9.83	98.9	67.0	79.1
9	susudu	4618	72.5	10.33	97.0	68.4	79.8
10	single ae	4582	84.6	15.67	xx	68.7	106.9
11	single wx	4654	72.5	16.17	xx	68.1	81.4
12	single su	4630	70.9	15.50	100.8	63.6	77.2
13	duduue	4588	71.8	13.50	100.5	67.1	81.1
14	duduwx	4666	71.9	10.17	98.8	67.2	79.5
15	dudusu	4642	71.7	10.17	97.2	66.8	79.0
16	single du	4606	71.6	9.50	100.1	65.8	79.8

Table 2

## 500 - Brabender Data

<u>Entry</u>	<u>Background</u>	<u>S93</u> <u>Row</u>	<u>Brabender</u>	<u>IR</u> <u>°C</u>	<u>HP</u> <u>BU</u>	<u>HF</u> <u>BU</u>	<u>CP</u> <u>BU</u>	<u>CF</u> <u>BU</u>
1	aeaewx	4660	460g/5.5%	65.0	315	285	940	940
2	aeaewx	4636	460g/5.5%	81.5	225	225	470	430
3	aeaewx	4612	460g/5.5%	84.5	180	180	410	380
4	wxwsae	4600	460g/5.5%	74.0	360	355	590	460
5	wxwssu	4648	460g/5.5%	74.0	325	325	540	470
6	wxwsdu	4624	460g/5.5%	80.0	315	270	480	425
7	susuae	4594	460g/5.5%	77.0	270	270	610	585
8	susudu	4672	460g/5.5%	77.0	295	295	785	610
9	susudu	4618	460g/5.5%	77.0	295	295	620	620
10	single ae	4582	460g/12%	90.5	170	170	240	240
11	single wx	4654	460g/5.5%	68.0	750	360	415	405
12	single su	4630	90g/5.5%	93.5	35	35	40	40
13	duduae	4588	460g/5.5%	83.0	225	225	545	520
14	duduwx	4666	460g/5.5%	86.0	245	245	600	550
15	dudusu	4642	460g/5.5%	84.5	270	270	620	585
16	single du	4606	460g/5.5%	89.0	70	70	160	160

IR = initial rise  
 CP = cooling peak  
 CF = cooling final

## 505

## - Brabender Data

<u>Entry</u>	<u>Genotype</u>	<u>Brabender</u>	<u>IR</u> <u>°C</u>	<u>HP</u> <u>BU</u>	<u>HF</u> <u>BU</u>	<u>CP</u> <u>BU</u>	<u>CF</u> <u>BU</u>
1	ae ae / wx	460g/5.5%	83.0	220	220	520	460
2	ae ae / wx	460g/5.5%	89.0	220	220	540	510
3	ae ae / wx	460g/5.5%	84.5	270	270	510	450
4	ae ae / ae	460g/12%	90.5	490	490	1220	845
5	su1 su1 / wx	460g/5.5%	80.0	250	240	595	535
6	du du / wx	460g/5.5%	83.0	280	250	560	470
7	du du / su1	460g/5.5%	50.0/83.0	230	230	575	535
8	fl fl / 0	460g/5.5%	81.5	280	255	630	560
9	su1 su1 / du	460g/5.5%	84.5	200	200	500	460
10	su1 su1 / ae	460g/5.5%	86.0	205	205	455	415
11	su1 su1 / su2	460g/5.5%	84.5	180	180	420	385
12	White waxy	90g/5.5%	68.0	830	220	310	270

Table 2

## - Starch Particle Size Data

<u>Entry</u>	<u>Background</u>	(Volume Distribution)				% Starch <u>Recovery</u>
		<u>S93 Row</u>	<u>Mode <math>\mu</math>m</u>	<u>Mean <math>\mu</math>m</u>	<u>Median <math>\mu</math>m</u>	
1	aeaewx	4660	16.90	12.57	16.04	45.9
2	aeaewx	4636	16.63	11.82	15.23	57.9
3	aeaewx	4612	16.36	11.48	15.20	52.4
4	wxwsae	4600	17.43	11.62	16.53	41.5
5	wxwssu	4648	17.72	12.43	16.59	58.8
6	wxwsdu	4624	17.48	12.22	16.26	54.1
7	susuae	4594	16.36	11.96	15.66	55.5
8	susudu	4672	16.65	9.83	15.29	58.7
9	susudu	4618	16.36	11.82	15.61	55.9
10	single ae	4582	13.09	9.07	12.25	64.0
11	single wx	4654	18.03	11.99	16.85	55.7
12	single su	4630	7.43	5.16	7.95	5.6
13	duduae	4588	16.90	11.01	15.37	50.9
14	duduwx	4666	17.19	11.85	16.21	52.5
15	dudusu	4642	17.17	12.22	16.01	44.9
16	single du	4606	14.41	9.77	13.19	58.9

510

## - Starch Particle Size Data

<u>Entry</u>	<u>Genotype</u>	(Volume Distribution)			% Starch <u>Recovery</u>
		<u>Mode <math>\mu</math>m</u>	<u>Mean <math>\mu</math>m</u>	<u>Median <math>\mu</math>m</u>	
1	ae ae / wx	16.63	10.90	15.70	51.4
2	ae ae / wx	16.12	10.44	14.92	52.8
3	ae ae / wx	15.60	10.55	14.60	59.4
4	ae ae / ae	11.53	8.40	11.30	63.7
5	su1 su1 / wx	14.88	10.24	14.21	52.5
6	du du / wx	16.38	10.73	15.12	52.0
7	du du / su1	15.87	11.03	14.98	53.2
8	fl fl / 0	16.12	10.73	15.35	69.3
9	su1 su1 / du	15.87	10.73	14.92	53.7
10	su1 su1 / ae	15.11	7.85	14.35	48.5
11	su1 su1 / su2	15.85	10.33	14.20	58.4
12	White waxy	17.19	8.45	14.81	71.5

515

Table 2

## - Starch Paste Data

<u>Entry</u>	<u>Genotype</u>	Brookfield Viscosity <u>CPS, 20 rpm</u>	Gel-24hr (grams)	Freeze Thaw Cycles
1	ae ae / wx	6,200	166.6	0
2	ae ae / wx	6,700	227.2	0
3	ae ae / wx	8,000	256.3	0
4	ae ae / ae	14,250	111.1	0
5	su1 su1 / wx	6,800	173.4	0
6	du du / wx	6,000	141.4	0
7	du du / su1	7,100	215.4	0
8	fl fl / 0	7,500	209.4	0
9	su1 su1 / du	6,300	219.7	0
10	su1 su1 / ae	6,000	172.2	0
11	su1 su1 / su2	5,500	158.8	0
12	White waxy	1,900	15.3	3

## - Starch Paste Data

<u>Entry</u>	<u>Genotype</u>	S93 <u>Row</u>	Brookfield Viscosity <u>CPS, 20 rpm</u>	Gel-24hr	Freeze Thaw Cycles
1	ae ae / wx	4660	25,000	151.8	0
2	ae ae / wx	4636	6,500	240.1	0
3	ae ae / wx	4612	3,150	216.6	0
4	ae ae / ae	4600	5,600	159.5	0
5	su1 su1 / wx	4648	6,100	126.4	0
6	du du / wx	4624	5,700	114.4	0
7	du du / su1	4594	8,700	254.8	0
8	fl fl / 0	4672	12,000	272.8	0
9	su1 su1 / du	4618	10,250	233.6	0
10	su1 su1 / ae	4582	2,500	53.7*	0
11	su1 su1 / su2	4654	3,100	13.3	0
12	White waxy	4630	275	29.0	3
13	duduae	4588	7,200	226.5	0
14	duduwx	4666	7,300	233.4	0
15	dudusu	4642	5,100	332.9	0
16	single du	4606	1,650	99.3	0

520

\* came out as a plug

## DEFINITIONS:

## DIFFERENTIAL SCANNING CALORIMETRY (DSC)

IR denotes initial rise

525 HP denotes heat peak

HF denotes heat final

CP denotes cooling peak

CF denotes cooling final.

## 530 BROOKFIELD VISCOMETER

The Brookfield Viscometer measures shear-strength (in centipoise, cP) and stability of starch pastes.

## BRABENDER VISCO-AMYLOGRAPH DATA

535 Pasting temperature denotes the temperature of paste formation.

Peak Viscosity denotes the temperature needed to provide a useable paste.

Viscosity at 95C denotes the ease of cooking of the starch.

Viscosity at 50C denotes the setback in paste viscosity during cooling of a hot paste.

540 Viscosity after 1 hour at 50C denotes the stability of the cooked paste.

## CORN PERCENT PROTEIN, STARCH, OIL AND MOISTURE

Percentages of oil starch and protein in corn give a measure of starch yield how recoverable the starch is.

545

## STARCH PERCENT PROTEIN, STARCH, OIL AND MOISTURE

Percentages of oil starch and protein in starch give a measure of how well purified the starch is and indicates millability.

## 550 PERCENT AMYLOSE AND L-MAX

These data provide a measure of apparent amylose levels in starch.



## STARCH PARTICLE SIZE DATA

Starch particle size gives an indication of starch yield and recoverability through  
 555 the milling process.

## SHORT HAND

aeaewx in table 2 refers to aeaeAE/wxWxWx, likewise  
 duduwx=duduDU/wxWxWx Throughout this table the wild type is not listed.

Figure 1 is a graph of enzyme activities for individual gene-dosages (e.g., MMM,  
 560 mMM, mmM, mmm) of mutant alleles of the single mutants of amylose extender  
 and dull. These data show the enzyme activities of sucrose synthase (SS), UDP-  
 glucose pyrophosphorylase (UDPG-PP), glucokinase (GK), fructokinase (FK),  
 phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), ATP-dependent  
 phosphofructokinase (PFK), PPI dependent phosphofructokinase (PFP), ADP-  
 565 glucose pyrophosphorylase (ADPG-PP), soluble starch synthase (SSS), branching  
 enzyme (BE) and bound starch synthase (BSS). Enzyme activities are presented as  
 percentages relative to the wild-type control (MMM). In the case of the full mutants  
 (mmm) there is a dramatic effect on expression levels of various enzymes in the  
 pathway of starch synthesis. In the case of the partial mutants (mMM and mmM)  
 570 there is almost no detectable change in expression levels. These data indicated that  
 the alteration in starch quality observed with the single mutants is a consequence of  
 the overexpression of several enzymes as well as elimination of the enzyme coded for  
 by the mutated allele. By combining two mutant doses (e.g., wxwxWx) with other  
 doses of another mutation (e.g., AeAeae) there would be partial reduction in two  
 575 enzymes without the overexpression seen in the rest of the pathway.

Figure 2 is a graph of the DSC scan of starches extracted from grain taken from  
 waxy, amylose extender and common (wild type) corn. Such DSC scans enable one  
 skilled in the art to provide numerical data (see tables in text for data on Peak  
 Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset  
 580 Temperature). It is particularly noteworthy that the profile of the high amylose starch  
 is different from the common starch and waxy starch.

Figure 2b is a graph of the DSC scan of starches extracted from grain taken from the double mutant (aeaeae/wxwxwx) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the double mutants is different from the data provided in Figure 2a on common starch and the single mutants, waxy and high amylose.

Figure 2c is a graph of the DSC scan of starches extracted from grain taken from intermutant (aeaeAe/WxWxwx) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the intermutant starch is different from the starch of the double mutant and appears to be similar to that of waxy starch.

Figure 2d is a graph of the DSC scan of starches extracted from grain taken from intermutant (wxwxWx/AeAeae) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the intermutant starch is different from the starch of the double mutant and appears to be similar to that of waxy starch.

Figure 3a is a graph of Brabender data taken from common starch in either neutral or acid conditions. Common corn starch shows substantial breakdown in viscosity using acid conditions.

Figure 3b is a graph of Brabender data taken from waxy starch in either neutral or acid conditions. The waxy mutation most particularly affects viscosity of the starch in neutral conditions.

Figure 3c is a graph of Brabender data taken from amylose extender (70% amylose) starch in either neutral or acid conditions. High amylose starches increase in viscosity in either acid or neutral conditions.

615

Figure 3d is a graph of Brabender data taken from double mutant (aeaeae/wxwxwx) starch in neutral conditions. Double mutant starches maintain viscosity despite being homozygous for the waxy mutation.

620

Figure 3e is a graph of Brabender data taken from intermutant (aeaeAe/WxWxwx) starch in neutral conditions. It is particularly noteworthy from these data that the new intermutant starches provide an increasing strength of viscosity similar to that seen with high amylose mutants, despite containing no increase in apparent amylose content.

625

Figure 3f is a graph of Brabender data taken from intermutant (wxwxWx/AeAeae) starch in neutral conditions. It is particularly noteworthy from these data that the new intermutant starches provide an increasing strength of viscosity similar to that seen with high amylose mutants, despite containing no increase in apparent amylose content.

630

### Example 2

This example illustrates the production of maize grain possessing starch of the present invention. Maize plants of various backgrounds can be converted to mutant genotypes using either traditional breeding and/or backcrossing techniques or else using mutagenesis such as chemical treatments of pollen. In the alternative, waxy inbreds and hybrids can also be purchased from a number of suppliers and foundation seed companies. Any maize line with good

635

640

645 agronomic traits and relatively high yield can be employed. In  
the present invention, normal inbred lines are converted to  
mutant inbred lines using chemical mutagenesis followed by  
careful selection of the mutant grain type from the segregating  
offspring. This method is well known to those skilled in the  
art (see for example, Neuffer, M.G. and Chang, M.T. 1989.  
Induced mutations in biological and agronomic research.  
Vortr. Pfalzenzuchtg. 16, 165-178). Any commercially  
650 valuable inbred line may be used for this process. The lines  
were confirmed to carry the mutation of interest by an allelism  
test in which the line may be crossed with a known mutant  
line, a process well known to those skilled in the art.  
Furthermore, the kernels from the line will have the  
655 appearance and iodine-staining characteristics typical of the  
mutation selected, a method well known to those skilled in the  
art. In order to obtain the highest yields from the plants it is  
best next to produce a hybrid cross between two inbreds, both  
inbreds carrying the same mutation (e.g., both inbreds being  
660 waxy or amylose extender types). It is preferred to produce  
two hybrids, one being the male and being homozygous for  
one mutation and the other being the female and being  
homozygous for the other mutation. The male and female  
hybrids can be made up of the same or different genetic  
665 backgrounds, it is merely important for the two lines to have  
similar maturities in the field (i.e., require similar heat units  
from germination to silking and pollen shed). In order to  
make the intermutant cross in the field it is necessary to  
eliminate pollen production from the female. This can be  
670 done by a variety of methods including, but not limited to,  
hand pollination, hand and mechanical detasseling,

introgressing genetic or cytoplasmic male sterility into the female plants, introducing male sterility through genetic transformation and use of chemical detasseling agents. The grain of this cross contains the present invention with a genotype in the endosperm of aaA/BBb, with the starch from those skilled in the art that the genetic background can be optimized for best starch qualities.

### Example 3

Starch may be extracted from grain by a number of different methods. The most commonly used method involves a "wet milling" procedure known and used throughout the world. The basic principle involves steeping and starch separation. The key step in this process involves softening the grain in a steep tank a process which has been optimized to permit optimal separation of the corn grain components. This method was employed to extract the starch, wxwxWx/AeAeae from the grain of an intermutant developed in accordance with Example 2. The germ was easily liberated intact and freed from adhering endosperm and hull. The endosperm is macerated under water, the starch was easily separated as a white floc and gluten proteins are obtained as a yellow floc. The grain was steeped for 30-40 hrs at 48-52°C in tanks usually holding 50-90 metric tons of grain. The steep water contains 0.2% sulphur dioxide (SO<sub>2</sub> gas is bubbled-in) and so is mildly acidic (pH 4.0). The sulphur dioxide helps break-up the protein matrix permitting the endosperm matrix to break-up into granules. After steeping, the grain were coarsely

705 ground or pulped. The oil-rich embryo floats to the surface  
and dense starchy endosperm sinks. Separation is achieved  
through hydroclones (continuous separation). The starch was  
710 purified further after being milled through "an impact mill  
known as Entoleter mill" which smashes the slurry at high  
speed through counter-rotating grooved plates made of  
hardened steel alloy, followed by impact with an outer impact  
ring. The defibred starch was separated from gluten by  
centrifugation to give two fractions: protein (70% protein) and  
715 starch (2% protein). Processing temperature was maintained  
above 45°C to prevent microbial growth. The starch was dried  
by flash-drying by injection into an air stream heated to 200-  
260°F.

720 Various intermutant grains can have the starch purified and  
prepared in this manner is suitable for a variety of food, feed  
and industrial uses. It may be used directly as unmodified corn  
starch. It may be modified by chemical or physical treatments  
that preserve granule structure and granules may be washed to  
remove residual reactants. Bleaching is sometimes used to  
create super-white starches. The starch can be gelatinized  
using a high temperature treatment and sold directly as  
gelatinized starch. Such starch may be chemically modified  
725 and dried. The polymer itself may be hydrolysed partially or  
completely to produce maltodextrins or glucose. Such  
products can be further modified by fermentation to produce  
ethanol for the gasoline industry, or the glucose can be  
converted to high-fructose corn syrup for the sweetener  
730 industry.

#### Example 4

735 The mutations called shrunken-2 (sh2), brittle-2 (bt2), dull (du), sugary (su), waxy (wx) and amylose extender (ae) encode isoforms of ADP glucose pyrophosphorylase, debranching enzyme, soluble starch synthase, bound starch synthase and branching enzymes:

740 Shrunken-2 encodes one subunit of ADP glucose pyrophosphorylase,

Brittle-2 encodes one subunit of ADP glucose pyrophosphorylase,

Waxy encodes granule bound starch synthase,

745 Amylose Extender encodes an isoform of branching enzyme, Dull alters expression of an isoforms of soluble starch synthase and branching enzyme,

Sugary alters expression and activities of soluble starch synthases and debranching enzyme.

750

Using known mutants and the gene-dosage crossing regimes we have examined the effects of altered gene expression on starch deposition in grain (See figures). With the bt2 mutant we see a progressive loss in measurable ADP Glc pyrophosphorylase activity which correlates well with a loss in starch synthesis in the grain. The control strength exerted by this enzyme over flux to starch cannot be quantified from these data. In fact our studies indicate that this enzyme is one of the major determinants of the duration of starch synthesis and may have little control over rate of starch synthesis. This mutation does not appreciably alter starch

755

760

765

770

775

780

structure. When the mutations are with sugary, dull, waxy and amylose extender we now do detect changes in starch fine structure (branched chain length changes as well as changes in amylose/amylopectin ratios). In these cases there is more minor control of flux to starch (except with the sugary mutant which is used to make sweet-corn genotypes). In all of these cases it is the changes in ratios of the starch synthases and branching enzymes which have resulted in alterations in starch fine structure. A dramatic new finding in these studies was the discovery that not only does the mutation reduce expression of key enzymes, but also it induces an overexpression of other enzymes in the pathway. Furthermore, it is only in the full mutant (mmm) genotypes where we see changes in starch fine structure demonstrating that the structural changes occur only when there is an enzyme isoform loss in combination with an enzyme isoform overexpression. Whilst not wishing to be bound by this proposal, these data illustrate the means by which starch structure may be influenced by not only reducing expression (eg using antisense constructs) an enzyme but also be simultaneously increasing expression (eg using sense construct).

785

790

Plant transformation vectors for use in the method of the invention may be constructed using standard techniques. Since these enzymes are localized in the amyloplast compartment of the cell, the gene construct requires the presence of an amyloplast transit peptide to ensure its correct localization in the amyloplast. The transformation construct may carry the gene either in the partial sense orientation or in the antisense orientation. Expression of said gene in the plant results in a reduction in expression of the enzyme by effects well known in the art as "sense cosuppression or antisense". When only a reduction in expression is needed the transit peptide is not



required. However, when enzyme overexpression is required then a correct plastid targeting sequence is needed in the construct. Key enzymes required for this invention include branching enzyme and soluble and bound starch synthase.

795 Branching enzyme [1,4- $\alpha$ -D-glucan: 1,4- $\alpha$ -D-glucan 6- $\alpha$ -D-(1,4- $\alpha$ -D-glucano ) transferase] converts amylose to amylopectin, (a segment of a 1,4- $\alpha$ -D-glucan chain is transferred to a primary hydroxyl group in a similar glucan chain) sometimes called Q-enzyme. Soluble starch synthase [ADPglucose:1,4- $\alpha$ -D-glucan 4- $\alpha$ -D-glucosyltransferase] extends the chain-length of amylopectin and perhaps also

800 amylose. Bound starch synthase [ADPglucose:1,4- $\alpha$ -D-glucan 4- $\alpha$ -D-glucosyltransferase] extends the chain length of amylose and perhaps also amylopectin.

For any antisense or sense-cosuppression construct only a partial cDNA clone is

805 required to be expressed in a transgenic plant. Where enzyme overexpression is required, then a full length cDNA clone is needed. The sequence of maize branching enzyme-I was investigated by Baba, T., Nishihara, M., Mizuno, K., Kawasaki, T., Shimada, H., Kobayashi, E., Ohnishi, S., Tanaka, K., and Arai, Y. (Identification, cDNA Cloning, and Gene Expression of Soluble Starch Synthase in Rice (*Oryza-sativa* L) Immature Seeds. *Plant Physiology*. 103:565-573, 1993). Starch branching enzyme-II from maize endosperm was investigated by Fisher, D.K., Boyer, C.D., and Hannah, L.C. (Starch Branching Enzyme-II from Maize Endosperm. *Plant Physiology*. 102:1045-1046, 1993). The article by Mu, C., Ham, C., Ko, Y., Singletary, G. W., Keeling, P. L. and Wasserman, B. P. shows an association of a

810 76kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. *Plant Journal* 6, 151-159 (1994). The maize waxy locus for UDP-glucose starch glycosyl transferase was cloned in 1986 by Kloesgen, R.B., Gierl, A., Schwarz-Sommer, Z. and Saedler, H (Molecular analysis of the waxy locus of *Zea*. *Mol. Gen. Genet.* 203, 237-244). Recently, the sequence for the maize sugary locus was observed by James, M. and Wright, A. (*The Plant Journal*) using

820 transposon mutagenesis to locate the gene. The gene for any such protein is thought

to be a debranching enzyme and may be used in constructs according to this invention.

825 It is believed that the chloroplast transit peptides have similar sequences (Heijne et al describe a database of chloroplast transit peptides in 1991, Plant Mol Biol Reporter, 9(2):104- 126). Other potential transit peptides are those of ADPG pyrophosphorylase (1991, Plant Mol Biol Reporter, 9:104-126), small subunit RUBISCO, acetolactate synthase, glyceraldehyde-3P-dehydrogenase and nitrite reductase. For example, the consensus sequence of the transit peptide of small subunit RUBISCO from many genotypes has the sequence:

MASSMLSSAAVATRTNPAQASM VAPFTGLKSAAFPVSRKQNLDTISIAS  
NGGRVQC

The corn small subunit transit peptide of RUBISCO has the sequence:

835 MAPTVMMASSATATRTNPAQAS AVAPFQGLKSTASLPVARRSSRSLGN  
VASNGGRIRC.

The transit peptide of leaf starch synthase from corn has the sequence:

MAALATSQLVATRAGLGVPDAS TFRRGAAQGLRGARASAAADTL  
SMRTASARAAPRHQQQARRGGR FPSLVVC.

840

#### Example 5

Production of fertile transgenic maize plants has been done since 1990. Although a number of DNA delivery systems are known, the selection is a particle bombardment. As noted above, constructs of the various maize mutant genes are available from depositories in the U.S. and Europe. Attached are a few examples of some of these constructs as shown in Figures 4a - d. Figure 4c shows a promoter, which is CaMv (cauliflower mosaic virus), an Adh1, the waxy gene, the nos(nopoline), and the pat gene which is useful as a selectable

845

850

marker and amp. Figure 4d is similar but shows the soluble starch syntheses first isoform gene in the construct. Figure 4a again has the same construct but shows the branching enzyme first isoform. Figure 4b shows the second branching enzyme second isoform. Of course, other constructs associated with the gene mutants used in maize breeding are also available.

For purposes of this example reference is made to Figure 4c, the waxy construct. The purpose of this experiment is to form an inbred that has partial down regulation of the waxy gene. If the inbred selected is already a mutant for ae, then the grain produced by crossing with a non-mutant inbred will be the grain of an intermutant. Depending on the strength of the down regulation, the female inbreds grain will resemble the mm\*/mm\* or the mm\*/m\*\* type of starch and grain. Clearly, the transformation allows a more precise way of down regulation of the starch synthesis activity such that the alteration of the starch can be finely tuned.

To assure reasonable levels of down regulation of the waxy gene, the transformation target tissue is immature zygotic embryos, through embryogenic callus can also be employed. Immature zygotic embryos from A188 plants 12 days after pollinated with the B73 ae inbred can be selected. The medium for the callus was 6 mM L-proline, 2% (w/v) sucrose, 2 mg/l 2,4- dichlorophenoxyacetic acid (2,4-D) and 0.3% (w/v) Gelrite (Caroline Biological Supply) (pH 6.0). Callus is grown and suspension cultures were initiated.

A MS-based liquid medium containing 100 mg/l myo-inositol, 2 mg/l 2,4-D, 2 mg/l 1-naphthalenacetic acid (NAA), 6 mM proline, 200 mg/l casein hydrolysate (Difco Laboratories), 3% (w/v) sucrose, and 5% (v/v) coconut water (Difco Laboratories) (pH 6.0). Cell suspensions were maintained in this medium in 125 ml Erlenmeyer flasks at 28°C in the dark on a gyrating shaker at 125 rpm.

The transformation vector in Figure 4c is selected. This plasmid contains a 35S-lacZ-pat nos 3' selectable gene expression cassette.

885 The cell suspensions are sieved and then suspended in 5 ml of suspension medium and placed on filter paper through vacuum. The construct was coated into particles as is known in the art. The plates were then bombarded. The cells are then transferred to a N-6 medium and after 14 days transformed cells are selected by 1 mg/l bialaphos. The cells are then suspended in a medium containing .6% (w/v) (Sea-  
890 Plaque; FMC) and held at 37°C.

Two to five weeks later, growing calli are removed and transferred to the surface of fresh selection medium. Plants were regenerated in a MS based medium having 6% sucrose 1g/l myo-inositol, 1 mg/l NAA (34), and 0.3% (w/v) Gelrite (pH 6.0).  
895 Next the embryo germination occurred in a MS media of 0.25 mg/l NAA and 3% (w/v) sucrose and light. Plants are grown and transferred to the greenhouse. The expression levels in the plant can then be evaluated.

The plant is bred and developed to an inbred having the mutant and the down regulated pathway. Alternatively, the selected inbred can have the mutant crossed onto a transgenic after transformation to form the desired starch in the grain when the transgenic plant is employed as the female.  
900

### Example 6

905

This example illustrates the gelatinization temperature of the starch of present invention compared to other starches. The gelatinization temperatures are listed in Table 3 below.

910

Table 3

<u>Starch Samples</u>	<u>% Amylose*</u>	<u>Gelatinization*</u>
		<u>Temperature °C</u>
1 Native common maize	28	71
2 AMY V native	57	80
3 AMY VII native	73	90
4 Present Invention	21	73
5 Native <u>ae wx</u>	25	80
6 Native <u>wx wx wx</u>	3	72

\*Values rounded to a whole number

915

Sample 1 was a commercial product sold by American Maize-Products Company of Hammond, Indiana. The percent amylose and the gelatinization temperature for Sample 1 above are mean values determined by random sampling of product. The 99% confidence level for percent amylose and gelatinization temperature are 25.9 to 29.3 and 68.7 to 72.9, respectively.

920

AMY V and AMY VII are commercial high amylose corn starches sold by American Maize-Products Company of Hammond, Indiana. The percent amylose and the gelatinization temperatures in Table 3 above are mean values determined from a random sampling of product. The 99% confidence interval for the percent amylose in AMY V and AMY VII was 53.4 to 62.5 and 65.5 to 73.8, respectively. The 99% confidence interval for the gelatinization temperature for AMY V and AMY VII was 72.8 to 84.4 and 83.1 to 90.8, respectively. Both AMY V and AMY VII were grown in native maize.

925

930

Starch Sample 4 corresponds to the Present Invention, while Sample 5 corresponds to the average values from Example 1 of U. S. Patent No. 5,009,911. Sample 6 corresponds to a commercial waxy starch sold by American Maize-Products Company.

The method for determining both the percent amylose and the gelatinization temperature was:

935 The percent amylose was determined using standard calorimetric iodine procedures wherein the starch is first gelatinized with sodium hydroxide and then reacted with an iodine solution and the resulting sample measured using a spectrophotometer in a 1 cm cell at 600 nm against a blank of 2% iodine solution.

940 The DSC gelatinization temperature was measured using a scanning calorimeter manufactured by Mettler Moddle No. 300 using 30% starch solids following the procedure outlined in the owner's manual for that model.

945 It is readily apparent from Table 3 above that the gelatinization temperature of the starch of the present invention is comparable to common corn starch.

#### Example 7

950 This example illustrates the gel strength of a sol made from the corn starch of the present invention compared to a sol made from æwx corn starch, a sol made from common corn starch, and a sol made from wxwxwx starch. The results of the test are reported in Table 4 below.

955 Table 4

<u>Sample</u>	<u>Strength (grams)</u>
Present Invention	159.5
Common Corn Starch	225.0
<u>æwx</u> Corn Starch	55.0
Waxy Corn Starch	16.0

In order to perform the gel strength test reported in Table 4 above, sols were prepared by mixing water with starch and subjecting the slurry to a rapid heat mode in the Brabender Visco-Amylograph to heat the sample to 50° C. Once 50° C was reached, the instrument was set at a controlled rate of heating, 1.5°C/minute, until a temperature of 95° C was reached. The sample was then held at 95° C for 30 minutes. Next, the sample was cooled at 1.5° C to a temperature of 50° C for 30 minutes. Portions of these sols were added separately to 4 ounce jars into which a plunger was placed. The sols were then allowed to stand at ambient conditions for 24 hours. Gel strength was measured by determining the force needed to remove the plunger from the sol.

This example illustrates that the gel strength of a sol made in accordance with the present invention is comparable to common corn starch sols.

970

#### Example 8

This example illustrates the difference between ae wx starch, a waxy starch wherein the plant had a triple dose of the waxy gene, and the starch of the present invention. All starches were obtained from maize.

975

All starches were tested for their rheological properties. Each starch was subjected to the same test procedure using the same method. The starch granules were pasting using a Brabender Visco-Amylograph with the cooling probe down and with the 750 g cm cartridge. The starch slurry, 5.5% initial solids, was rapidly heated to 60° C in the Brabender cup, and then pasted while increasing the temperature to 95° C at 1.5° C per minute. The starch paste was held at this temperature for 20 minutes, and then immediately loaded onto the measuring geometry of the rheometer which had been preheated to 70° C. A four-part rheological characterization was performed. The gel

980

985

cure segment which monitored the formation of structure at .2 hertz and .2% strain (well within the linear viscoelastic region) was measured as the sample was cooled from 70° C to 25° C and held for 4 hours. Also measured was the strain sweep, which measures the rheological response of the paste or gel to increasing levels of strain, also at 0.2 hertz, at 25° C.

Using this technique, distinct differences between ae wx starch and the starch of the present invention were found. Figure 5 shows the results of the gel cure analysis. The starch of the present invention, while having an initial Modulus (G') lower than ae wx starch, more quickly formed structure or gelled as evidenced by the rapid rise in G'. Thus the starch of the Present Invention was distinct from ae wx starch in the rate of gel formation despite similar apparent iodine binding contents. Figure 6 shows the results of the strain sweep analysis only for the starch of the present invention and the ae wx starch. Here the starch of the present invention showed dilatant behavior as evidenced by the increase in G' as the strain level was increased. Under the applied levels of strain, the structure was not destroyed. In contrast, the structure of the ae wx starch was rapidly destroyed when the applied strain became greater than 4%. Thus, when compared to ae wx starch, the starch of the present invention formed a gel which didn't break under the applied strain of this test. In contract, ae wx starch showed much less time-dependent structure formation and did "break" or was destroyed by the strains applied in this technique.

#### Example 9

This example illustrates preparing a thickener composition in accordance with the present invention.

The starch of the present invention is mixed with water in an amount to produce a slurry having 10% by weight starch. The sol has a short texture and a bland taste. The sol when cooked at about 90° C for 10 minutes produces a thickener



composition which had better clarity than a similar thickener composition made from common corn starch and a shorter texture.

### Example 10

1020

This example compares the mouth feel of a gel made from the starch of the present invention to a gel made with a common starch.

1025

The common starch and starch of the present invention were pasted using a Brabender visco-amylograph. The starch was slurried at 5.5% solids, and then heated using the rapid heat mode to 50° C. Using controlled heat of 1.5° C per minute the slurries were heated to 95° C, and then held at this temperature for 30 minutes. The final solids was 5.9%. The sample starch pastes were then poured into small jelly

1030

jars, covered with cellophane, and allowed to age 24 hours before analysis. A taste panel was then asked to rank the samples for the following attributes.

First they ranked the two for relative firmness to the touch.

#### Common

5.1

#### Present Invention

5.6

1035

Next, they ranked the relative break, firmness, and clearing of this sample while being masticated.

	<u>Common</u>	<u>Present Invention</u>
Degree of clean break	3.2	8.5
Firmness	4.4	4.5
Rate of clearing	2.5	4.6

These results show that the firmness of these samples is similar. However the  
1040 starch of the present invention has a much cleaner break while being masticated.

Furthermore, it tends to clear from the mouth faster than a common based starch  
gel. The panel all agreed that the starch of the present invention produced a gel  
which had a "clean" mouth feel similar to that of a gelatin or a pectin.

1045

### Example 11

This example illustrates making a gum candy using the starch  
of the present invention.

1050

The following ingredients and procedure are used:

Table 5

<u>Ingredients</u>	<u>% by Weight</u>
	<u>Present Invention</u>
44/62 Corn Syrup Unmixed	56.34
Sugar, fine granular	23.98
Water	7.73
Present Invention Starch	11.80
Citric Acid	0.07
Sodium Citrate	<u>0.08</u>
	100.00

### Procedure

1055

All ingredients are mixed and then cooked to 340° F using  
conventional equipment such as a jet cooker. The cooked  
slurry is then poured into candy molds and allowed to solidify.

**Example 12**

1060

This example illustrates making a Bavarian cream pie using starch of the present invention.

The following ingredients and procedure are used:

1065

**Table 6**

<u>Ingredients</u>	<u>% by Weight</u>
	<u>Present Invention</u>
Whole milk, fresh 3.5%	72.794
Sugar, fine grain	17.586
Salt, Flour	0.101
Present Invention	5.410
Banana Flavoring	0.300
Egg Yolk, fresh	<u>3.809</u>
	100.000

**Procedure**

1070

All of the pie filling ingredients except for egg yolks are combined and cooked at 195° F for 3 to 5 minutes. Then the ingredients are cooled to 120°F with constant stirring. Next, egg yolks are added and the admixture well blended. This mixture is then added to a conventional pie crust and allowed to cool to room temperature before serving.

1075

**Example 13**

1080

This example illustrates preparing a lemon pie filling with the starch of the present invention.

The following ingredients and procedure are used:

**Table 7**

<u>Ingredients</u>	<u>% By Weight</u>
	<u>Present Invention</u>
Water	62.94
Sugar	19.30
Maltodextrin	6.67
Present Invention	4.50
Corn Syrup Solids	2.50
Lemon Juice	2.50
Vegetable Shortening	1.03
Salt	0.23
Citric Acid	0.20
Emulsifier	0.10
Lemon Oil (2x)	<u>0.03</u>
	100.00

1085

**Procedure**

1090

Half of the water is combined with the sugar and brought to a boil. All of the remaining ingredients are slurried together and then added to the boiling sugar and water. The temperature of this mixture is then adjusted to 200° F. and held there for two minutes. The mixture is then poured into prepared pie crusts and allowed to cool and solidify.

**Example 14**

1095 This example illustrates making a chocolate mousse using the starch of the present invention. The formulation in Table 8 is employed to prepare a mousse mix.

**Table 8**

1100

	<u>%</u>
Frodex 24-924	39.20
Sugar (Baker's)	30.75
Whiptreme 3554 (Kerry Ingredients)	12.88
Starch of Present Invention	9.80
Cocoa, Dutch Red (Gill & Duffus Products)	7.17
Leceitreme 40 (Kerry Ingredients)	0.20
Flavor	<u>as desired</u>
	100.00

Procedure

Combine ingredients to form a uniform blend.

1105

Use

Combine 200 gms. of mousse mix with 1 cup (250 grs.) milk. Using an electric mixer, combine on low speed for 1 minute. Scrape bowl. Mix on high speed for 3 minutes, until light and fluffy. Spoon into serving dishes and refrigerate for 1 hour before serving.

1110

To prepare the mousse mix, all the ingredients are mixed. To prepare the mousse itself, 200 grams of mousse mix are

1115

combined with 250 grams of milk and combined at a low speed. Then the mixture is stirred at a high speed to make it light and fluffy and the mixture is refrigerated for one hour. In this way, a light, fluffy mousse is prepared.

1120

Accordingly, the present invention has been described with some degree of particularity directed to the preferred embodiment of the present invention. It should be appreciated, though, that the present invention is defined by the following claims construed in light of the prior art so that modifications or changes may be made to the preferred embodiment of the present invention without departing from the inventive concepts contained herein.

1125